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14. ABSTRACT

Multicellular tumor spheroids are a suitable tumor model system for investigating the effect of anti-cancer agents on tumor growth. The overall objective of the present investigation is to examine the effect of isolated eosinophil toxic granular protein(s) and cytokines IL-4, IL-10, IL-12, TNFα on markers of tumor growth and metastasis (e.g. erbB2, cyclin D1, cyclin E). Early studies concentrated on the development of the spheroid model. Optimization of spheroid size, shape and number was emphasized in year 1. Of the two non-metastatic breastcell lines selected, the MCF-7 tumor line formed ideal spherical tumors, while the T-47D cells took longer to form round spheroids which were half the size of the MCF-7 MTS. The metastatic cell line MDA-MB-468 fared to grow. The data are inconclusive regarding proliferative state of the MTS fractions. They were too few cells in the early fractions. All eight eosinophil cell lines were examined and ready for batch culture for protein isolation. Additional experiments are needed for optimal standardization of the MTS models.

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3. Introduction

Multicellular tumor spheroids (MTS) represent an *in vitro* model of solid tumor nodules. As these tumors grow the central portion (core) becomes compromised by lack of oxygen and nutrients. This is primarily due to the avascular nature of the inner tumor (1-3). The radially symmetric growth of the spheroids results in the formation of distinct zones of decreasing proliferation from the highly proliferative outer rim to the inner necrotic core (4,5). These quiescent nonproliferative zones mimic that observed in tumors *in vivo* (4).

The hallmarks of cancer have been summarized into seven critical characteristics: 1) growth signal controls, 2) resistance to anti-growth signals, 3) evasion of apoptosis, 4) uncontrolled replicative potential, 5) sustained angiogenesis, 6) invasion and metastasis and 7) genomic instability (6). The MTS model system offers an excellent tool for examining the impact of chemotherapeutics on these seven areas of cancer growth and development.

We have studied the eosinophil as an anti-cancer effector cell and have demonstrated its inhibition of breast tumor cell growth (7). Moreover we have demonstrated eosinophilic infiltration of MCF-7 MTS and release of granular proteins (8). In this study we will examine the effect of isolated eosinophil granular protein (s) on four of the seven markers or characteristics of tumor growth (growth control signals, resistant anti-growth factors, evasion of apoptosis, and invasion and metastasis) in various proliferative and quiescent zones of MCF-7 MTS. Specifically, the expression of the following markers will be determined: erbB2, cyclin D1, cyclin E, cyclin kinase inhibitors, p21 and p27, p53, adhesion molecules E-cadherin and N-cadherin. We will also examine the effect of proinflammatory and non-inflammatory cytokines (pre-formed in eosinophil granular cores) alone and in combination with eosinophil granular protein (s) on the expression of these markers of cancer growth and proliferation.

4. Body

Eosinophil cell lines. Eight eosinophil cell lines (hypodense and hyperdense) from 4 individuals with varying degrees of allergy/asthma severity and therefore with varying levels of eosinophilia. The list of cell lines are as follows: LAE.013.22, LAE.013.24, GRC.014.22, GRC.014.24; CTA.052.22, CTA.052.24; BJA.060.22, BJA.060.24. The .22 and .24 stems represent cell lines established from hypodense and hyperdense eosinophils, respectively.

Tumor Cells. Tumor cells selected for this study include: MCF-7, T47-D (ER^+), BT-549, MDA-MB-468 (ER^-) and the non-tumor line, MCF-10A. All cells were purchased from

American Type Culture Collection, (Manassas, VA). The cells were propagated in the recommended culture media: ***MCF-7*** – RPMI complete medium with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate, supplemented with 0.01 mg/ml bovine insulin, fetal bovine serum, 10%; ***T-47D*** – RPMI medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, supplemented with 0.2 Units/ml bovine insulin, and fetal bovine serum, 10%. ***BT-549*** – RPMI complete medium (same as for T-47D); ***MDA-MB-468*** – Leibovitz's L-15 medium with 2 mM L-glutamine, and fetal bovine serum, 10%; ***MCF-10A*** – Mammary epithelial growth medium (MEGM), serum-free, supplemented withy 100 ng/ml cholera toxin.

MCF-7 Multicellular Tumor Spheroids. MCF-7 multicellular tumor spheroids were developed by modifying the method of Yuhas *et al*. and Freyer *et al* (9, 10). Briefly, subconfluent monolayer cultures, (maintained at 37°C, 100% relative humidity, 95% air, 5% CO_2) in the appropriate culture media was used to prepare MTS. After trypsinization, the cells were counted and resuspended to a concentration of $5x10^4$/ml. 200µl of the resuspended cells ($5x10^4$ cells/ml) were seeded into 96 well U-bottom plates containing an overlay of 1.5% Ultra Pure agar using a multiwell pipetter. The 96 well plates were then incubated at 37°C in a 5% CO_2 atmosphere for 2 days. On day 3 100µl of the culture media was removed and fresh media was added. On day 7, the formed spheroids were removed from the 96 well plates and introduced into a Bellco spinner flask to promote further growth and increase in size and diameter. Media in the spinner flask was replenished daily until spheroids were used for experimental testing (day 10). Diameter measurements were made with an ocular micrometer on a Zeiss Axiovert 100 inverted phase contrast microscope.

Dissociation of Spheroids

Spheroids dissociations were performed by first removing the spheroids from the spinner flasks and washing with PBS. The spheroids were then placed in ice cold PBS until dissociated. The dissociation system was first flushed with a 10% bleach solution followed by flushing with PBS for 5 minutes. Following the flushing of the dissociation system, the spheroids were transferred to the dissociation chamber using a 5 ml pipette. The tubing was transferred from the PBS bottle to the trypsin bottle, and the residual PBS flushed from the system. The spheroids in the dissociation chamber were exposed to a continuous flow of trypsin (0.025%). The cells that eluted from the dissociation chamber were collected at 3 minute intervals in Erlenmeyer flasks containing cold medium with 10% FBS to stop the action of trypsin. After collecting the last fraction, the pump was stopped and the remaining contents were transferred to the last flask and the necrotic core of cells was suspended pipetting. The contents from each Erlenmeyer flask were

transferred to 50ml tubes, centrifuged, resuspended in fresh medium and counted. After performing the cell count on each of the 12 fractions collected, the cells in the 50ml tubes were combined to form 4 fractions. Each of the 4 fractions represents a zone of decreasing proliferation (zone 1, the highly proliferative outer rim to zone 4, least proliferative inner zone nearest to the necrotic core (5). These cells were later subjected to RNA isolation to evaluate gene expression.

Immunohistochemistry. Cytospin slides of the various dissociated spheroid fractions of the MCF-7 multicellular tumor spheroids were prepared. Detection of Ki67 nuclear antigen (proliferation marker) was performed using the monoclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Slides were incubated with 1X PBS/1% BSA/1% NGS for 60 min at room temperature to prevent non-specific binding, then incubated overnight with primary anti-Ki67 monoclonal antibody, washed 3X , then incubated with secondary (FITC-conjugated goat anti-mouse IgG) for 2 hrs at room temperature. The slides were scored by visual examination, using fluorescent microscopy.

Eosinophil Cell Line (s) in Culture. Eight eosinophil cell lines, GRC.014.22, GRC.014.24, BJA.060.22, BJA.060.24, CTA.052.22, CTA.052.24, LAE.013.22, and LAE.013.24 were retrieved from liquid nitrogen storage, propagated in culture, and examined for growth characteristics. Samples from each line were frozen and stored in liquid nitrogen. Figure 1 is representative of the morphological growth pattern of these cells in culture. These cells will be batch-cultured for the isolation of eosinophil granular protein, major basic protein. Figure 1 is a photomicrograph of GRC.014.22 in culture. All eosinophil cell lines grow similarly.

Tumor cell lines. Photomicrographs of T-47D, MCF-7 (ER^+), BT-549 (ER^-) and a non-tumor line, MCF-10A monolayer cultures (Figure 2). The fourth tumor line, MDA-MB-468 failed to grow in our laboratory and we will be purchasing another metastatic ER^- cell line in the near future.

Schematic of development of multicellular spheroids. Multicellular spheroids form in 96 wells and are transferred to spinner flasks to increase in size and growth. The spheroids are then placed in the dissociation chamber and subjected to trypsin. Cells are collected every three minutes (Figure 3).

MCF-7 24hr Multicellular Tumor Spheroid (MTS). 24 hr after growth in 96-well round bottom culture plates photomicrographs were taken (Figure 4).

T-47D 24hr Multicellular Tumor Spheroid (MTS). T-47D spheroids also formed within 24 hr of culture of cells in 96-well plates. The size of these spheroids at this time were half that of the MCF-7 (700 um vs 1400 um) (Figure 5).

MCF-7 48hr Multicellular Tumor Spheroid (MTS). On day 2, the MCF-7 spheroids increase in size and continue to form tight compact nodular-like tumors. (Figure 6).

T-47D 48hr Multicellular Tumor Spheroid (MTS). T-47D spheroids, misshapen at 24 hr, become more spherical at 48 hrs. The size differential remained half that of the MCF-7 spheroids (Figure 7.).

MCF-7 Multicellular Tumor Spheroids (MTS) Pre-Spinner Flask. Spheroids are allowed to grow in the 96-well culture plates for 7 days before transferring them to a spinner flask for continued growth. Figure 8 illustrates a MCF-7 spheroid with necrotic core that is dispensed into spinner flasks.

MCF-7 MTS 48 hr in Spinner Flask. The spheroids continue to grow in the spinner flask for an additional three days. Figure 9 captures an MCF-7 spheroid after 48 hr in culture.

MCF-7 MTS at Day 10 (Pre-Dissociation). Large spheroids with clear distinct necrotic sores are used in the dissociation step. Figure 10 is an example of a large MCF-7 spheroid that is transferred to the dissociation chamber.

MCF-7 MTS During Dissociation. Figure 11 shows a representative spheroid after 10 three minute dissociation steps. The spheroid is still intact.

MCF-7 MTS Necrotic Core. Figure 12 shows a photomicrograph of the necrotic core after 14 dissociation steps.

RNA Concentrations of Dissociated Spheroid Zones. The dissociated fractions of cells were combined into 4 major fractions (zones) as described by Freyer, representing zones of proliferation.

Ki-67 Expression in Fractions of MCF-7 MTS. Cytospin slides were prepared from spheroid dissociated fractions.

5. Key Research Accomplishments

- Retreival of all eight eosinophilic cell lines to be used to isolate granular protein
- Propagation of 3 of 4 of the breast cell lines and the non cancer MCF-10A cell line
- Growth of MCF-7 multicellular tumor spheroids, dissociation and RNA quantitation of proliferative zones
- Evidence of Ki-67 proliferative marker in spheroid dissociation fractions

6. Reportable Outcomes

Ph.D. dissertation research advisor:

Ph.D. dissertation committee member:

Agnes B. Baffoe-Bonnie, Rick A Kittles, Elizabeth Gillanders, Liang Ou, Asha George, Christiane Robbins, Chiledum Ahaghotu, James Bennett, William Boykin, Gerald Hoke, Terry Mason, Curtis Pettaway, Srinivasan Vijayakumar, Sally Weinrich, Mary P. Jones, Tracy Moses, Erica Lockwood, Mechan Klaric, Mezbah Faruque, Charmaine Royal, Jeffrey M. Trent, Kate Berg, Francis S. Collins, **Paulette Furbert-Harris**, Joan E. Bailey-Wilson, Georgia Dunston, Isaac Powell, and John D Carpten. Genome-Wide Linkage of 77 Families From the African American Hereditary Porstate Cancer Study (AAHPC). The Prostate April, 2007 67:22-31.

Mathias RA, Gao P, Goldstein JL, Wilson AF, Pugh EW, **Furbert-Harris P,** Dunston G, Malveaux F, Togias A, Barnes KC, Beaty TH, Huang SK A graphical assessment of p-values from sliding window haplotype tests of association to identify asthma susceptibility loci on chromosome 11q. BMC Gent. 2006 June 14;7(1):38.

7. Discussion/Conclusions

The overall objective of the study is to determine the effect of eosinophil granular protein, MBP, alone and in combination with proinflammatory and noninflammatory cytokines on the expression of cancer markers involved in regulating growth and proliferation, resistance to apoptosis, invasion and metastasis. The tasks of the first 12 months were as follows:

a. retrieval and propagation of eight eosinophil cell lines (hypodense and hyperdense) in preparation for bulk culture.

b. Purchase and propagation of 4 breast cancer cell lines and one non-cancer cell line.
c. Baseline studies with spheroid generation and determining the number of spheroids needed for RNA analysis.
d. Immunochemistry with dissociated spheroid fractions for the detection of Ki-67 proliferation marker.

The tasks of year 1 have been met as it pertains to acquisition and retrieval of both tumor and eosinophil cell lines. We were unsuccessful in growing the metastatic cancer cell line MDA-MB-468 and we will be substituting another. The growth of multicellular spheroids was accomplished with both MCF-7 and T-47D cell lines. Complete dissociation , however, was performed with only the MDF-7 cell line as this cell line generated the largest spheroids with optimum integrity . Moreover, because of its smaller size and no central core, T-47D spheroid generation was discontinued at this time. The number required for suitable concentrations of RNA from each zone has been determined. A total of 5 96-well plates were used to generate spheroids. The efficiency of generation was 90%, therefore approximately 418 spheroids were dissociated per experiment. Each experiment lasted 10 – 13 days (i.e., up to RNA isolation). The immunohistochemistry staining for the proliferative marker, Ki-67 was incomplete because there were insufficient cell numbers in the early fractions. Therefore the data generated thus far were equivocal. Additionally, assay staining conditions (antibody concentrations) need to be further standardized. A greater number of spheroids will have to be generated in order to obtain sufficient cells in the early fractions for staining. Spheroids from both metastatic and nonmetastatic, as well as the non tumor control, will be treated with eosinophil granular protein and cytokines, then dissociated in order to examine gene expression in zones of quiescent cells.

8. References

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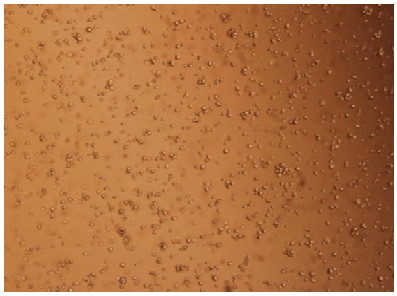
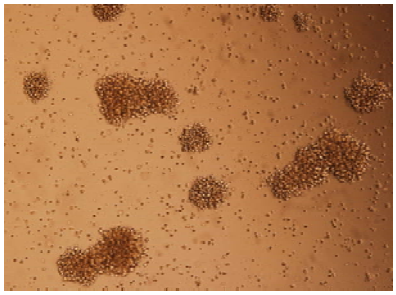
9. Appendices

Supporting Data:

Figure 1. Eosinophil Cell Line



Eosinophil Cell line at Initial Seeding

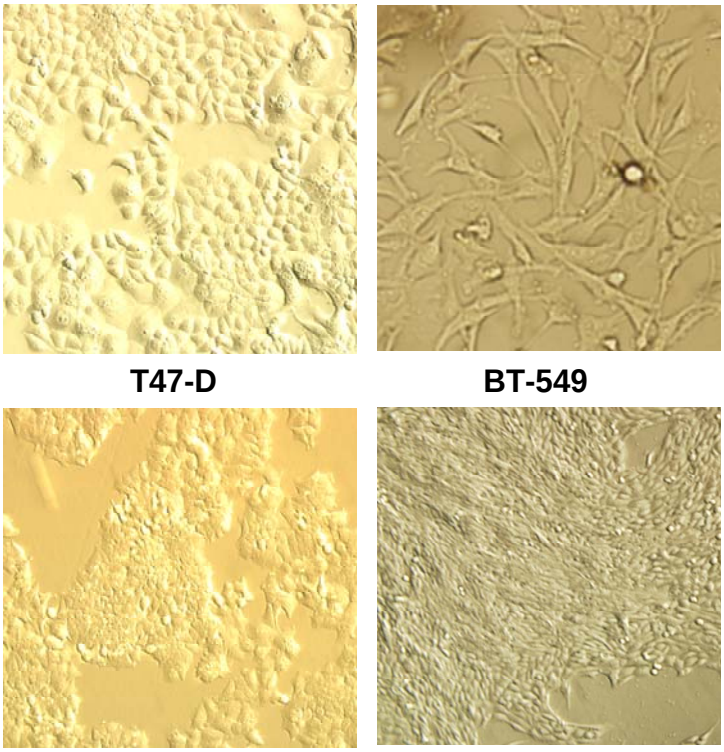


Eosinophil Cultures After 4 Days of Culturing

GRC.014.22. GRC.014.24
BJA.060.22; BJA.060.24
CTA.052.22; CTA.052.24
LAE.013.22; LAE.013.24

Figure 1. ***Eosinophil cell Lines in Culture.*** Eosinophil cell lines were retrieved from liquid nitrogen, cultured and frozen off to ensure that these cells would be available for use. Photomicrographs of cell line GRC.014.22 were taken (day 1 and day 4). All eosinophil cell lines had the same growth characteristics.

Figure 2. Breast Tumor Cell Lines



T47-D **BT-549**

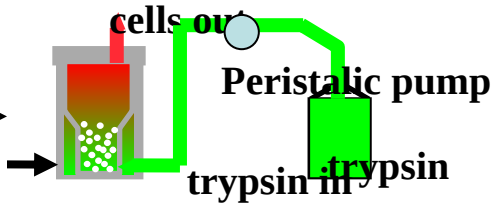
MCF-7 **MCF-10A**

Figure 2. Tumor Cell lines. Three tumor cell lines, MCF-7, BT-549, and T-47D and one non-tumor breast lines were grown in culture. Frozen samples were made and two of the lines were continued in culture for spheroid growth.

Figure 3.

On day 1 200 ul cells ($5x10^4$ /ml) are dispensed into each well [wells precoated with agarose (1%)]. The plates are incubated at 37C. The plates were replenished with fresh medium every other day. Cells form multicellular spheroids within 72 hrs.

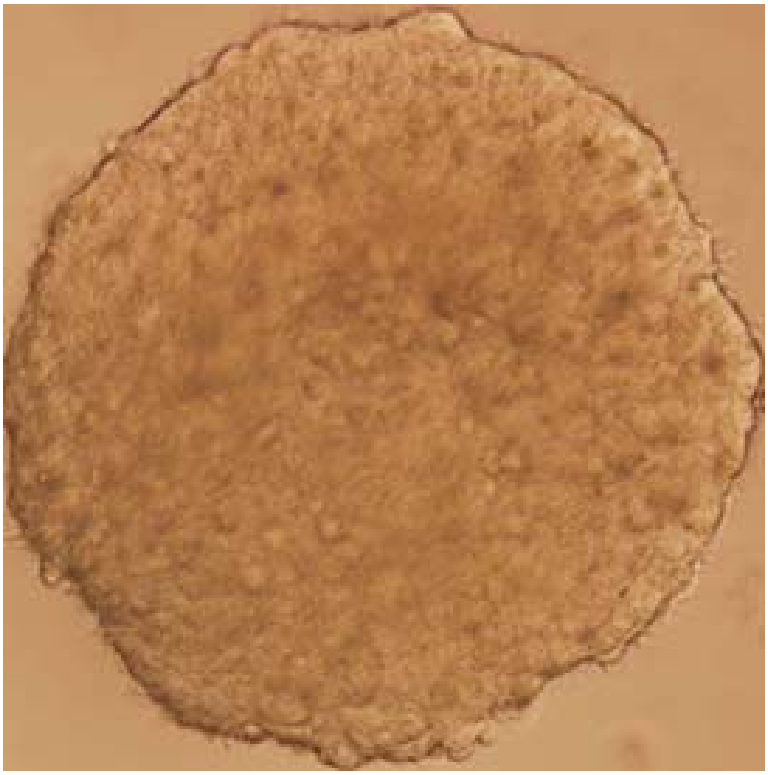
On day 7 Intact spheroids are placed into the Spinner flask which is cultured for an additional 3 days. The spheroids are fed every day at this stage.



Schematic of the spheroid dissociation chamber. Large arrow points to spheroids within the chamber. Trypsin (0.025%) is pumped into the chamber, interacting with the swirling spheroids, dissociating single cells from the spheroid mass. The larger heavy spheroids remain in the lower chamber, while the dissociated lighter single cells move to the top and are collected.

Figure 3. Schematic of Multicellular Tumor Spheroid Generation. 200 ul of tumor cells ($5x10^4$/ml) were dispensed into 96-well round bottom culture plates (with agar overlay). The spheroids (7-day) were transferred to spinner flasks and cultured for an additional 3 days, then collected and transferred to the dissociation chamber, then subjected to 3-minute trypsin treatment (continuous flow).

Figure 4.

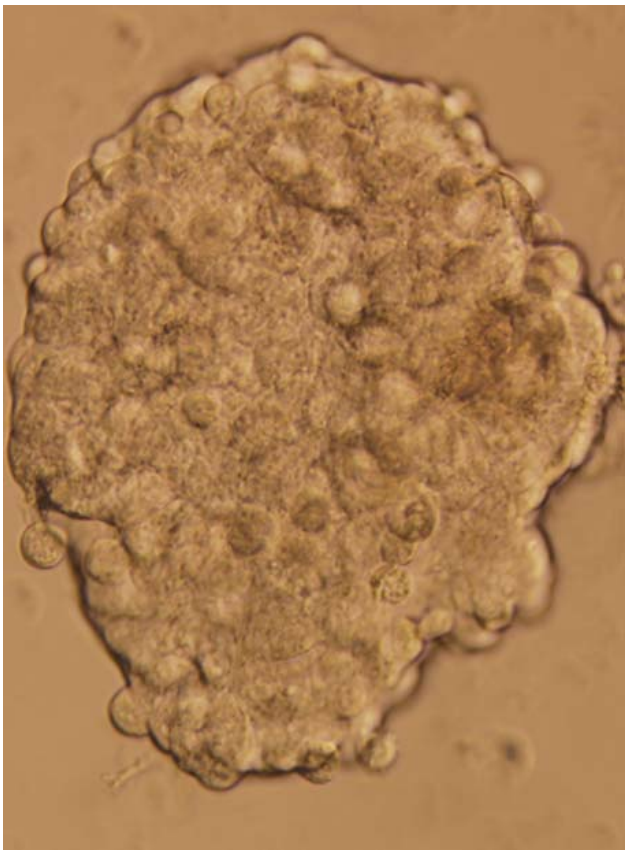


MCF-7 MTS (1400um) 4X mag.

24 hr Culture

Figure 4. MCF-7 multicellular spheroid post 24 hr culture in 96-well plate.
Spheroids form within 24 hrs culture in 96 well culture plates that were overlayed with agarose (1.5%).

Figure 5.

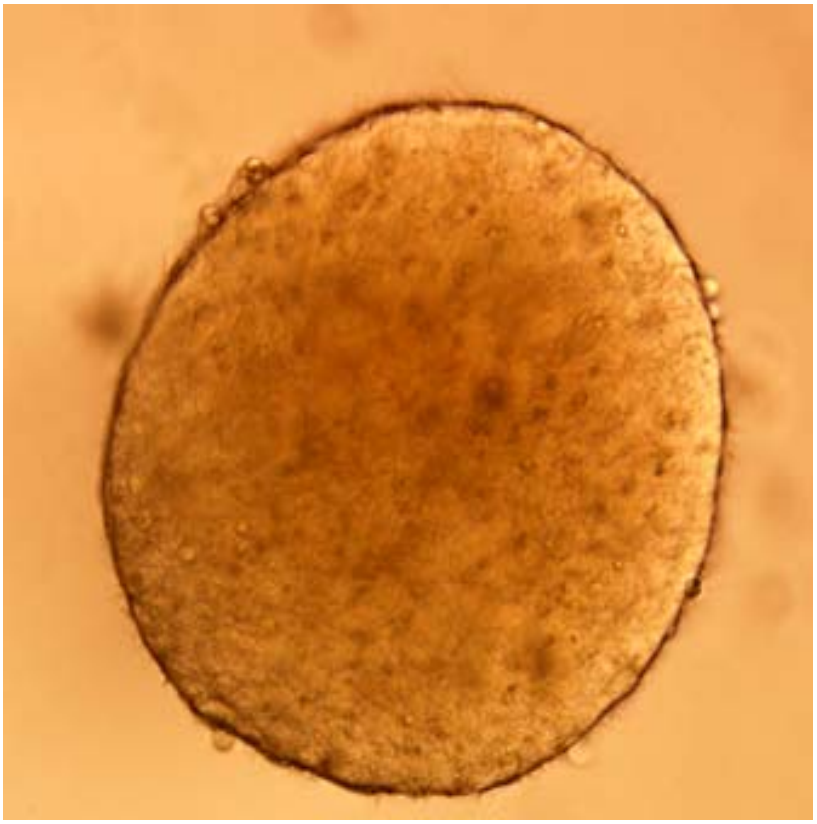


T-47D MTS (700 um) 10x mag.

24 hr Culture

Figure 5. ***T-47D multicellular spheroid 24 hr post seeding 96-well culture plate.*** Unlike the MCF-7 spheroids, T-47D spheroids were irregular in shape at 24 hr and also much smaller**.**

Figure 6.



MCF-7 MTS (48 hr)

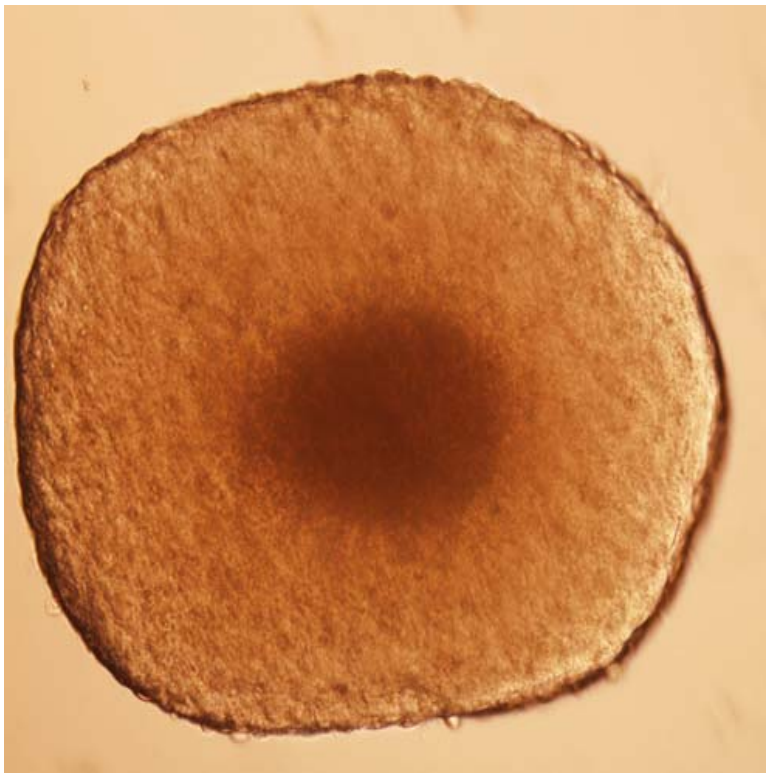
Figure 6. MCF-7 multicellular spheroid 48 hr in 96-well culture plate. The spheroids continue to grow and become round. The integrity of the spheroid is such that the borders are almost completely smooth, with the appearance of only one or two single cells.

Figure 7.

T-47D MTS (48 hr)

Figure 7. ***T-47D multicellular spheroid 48 hr in 96-well culture plate.*** Although the spherical shape begins to take form at 48 hr, the borders still remain rough.

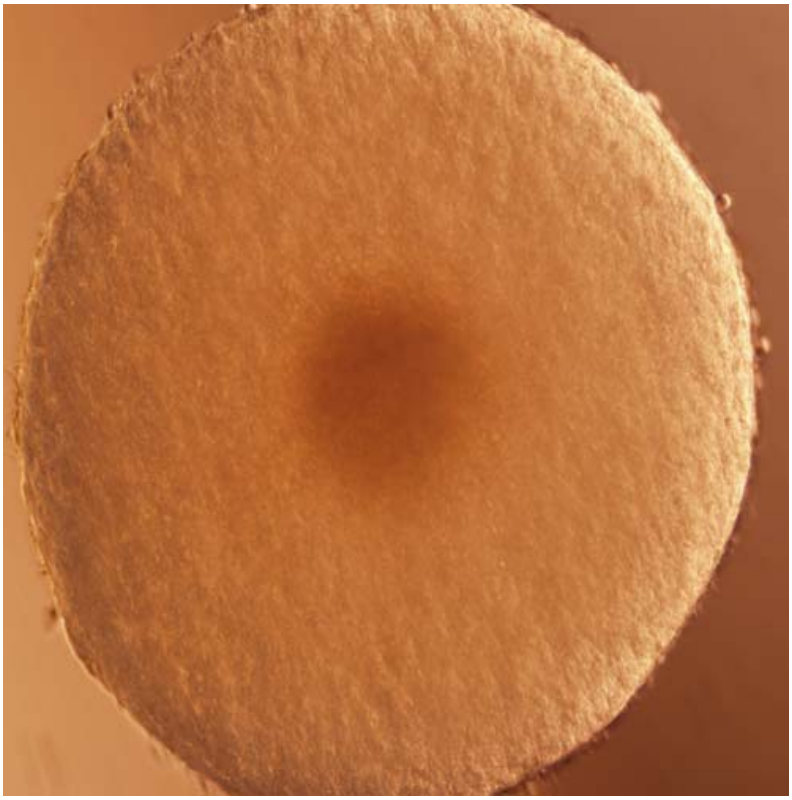
Figure 8



MCF-7 MTS Pre-Spinner Flasks

Figure 8. MCF-7 spheroids pre-spinner flask. At day 7, the fully mature MTS is transferred to the spinner flasks to allow them to increase in size. Figure 8 is a photomicrograph of an MCF-7 spheroid with its characteristic necrotic core that is transferred to the spinner flask.

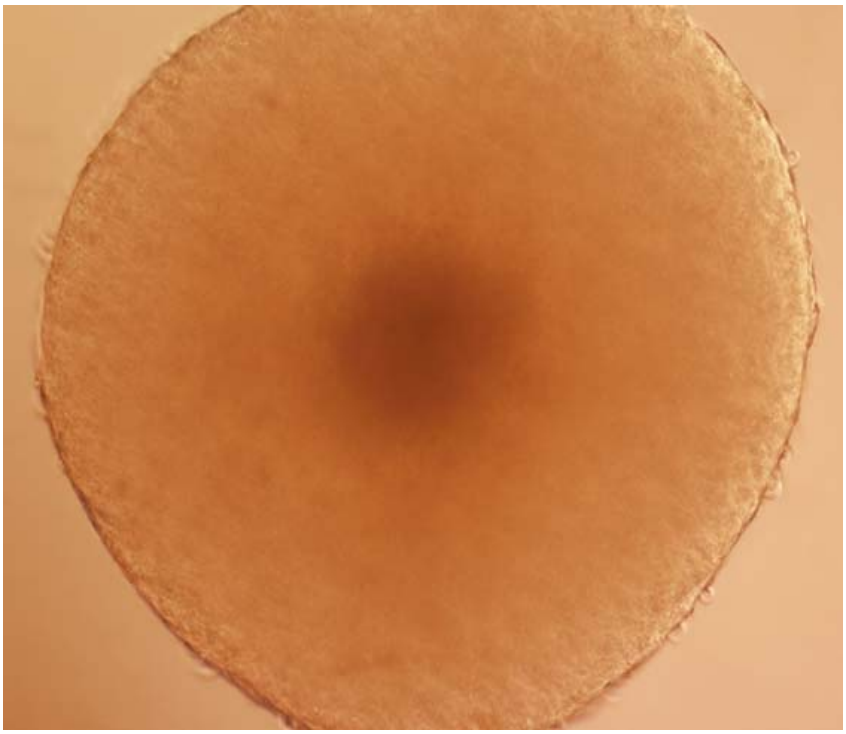
Figure 9.



MCF-7 MTS at 48 hr in Spinner Flask

Figure 9. MCF-7 spheroids after 48 hr in spinner flask. The spheroid continues to grow in size, forming the characteristic gradient of proliferative (outer zones) and nonproliferative quiescent (inner zones near necrotic core) cells.

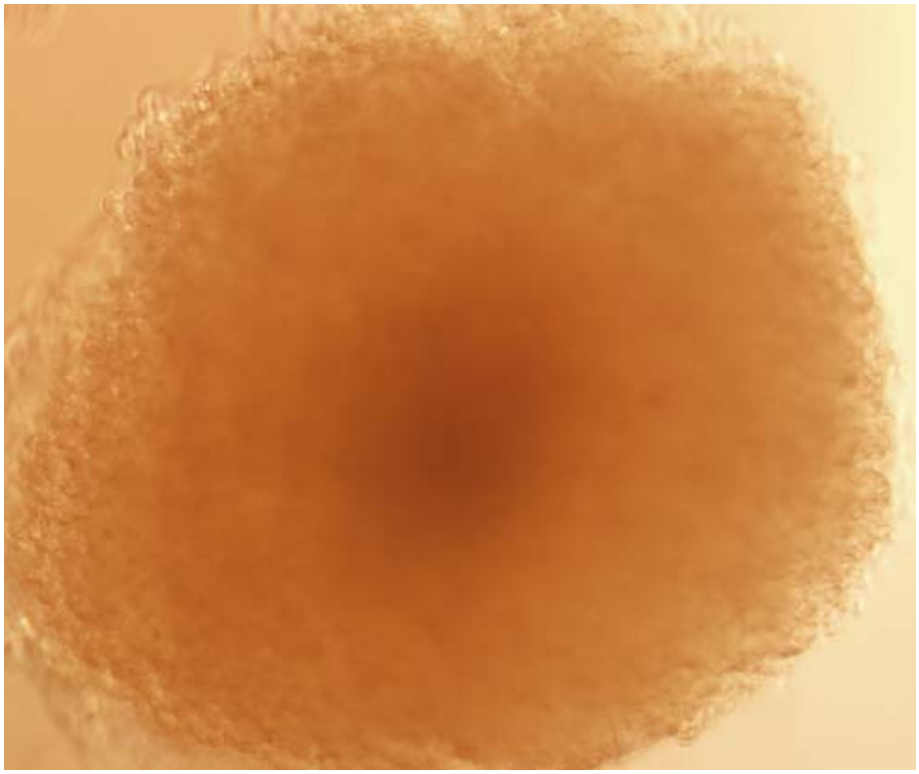
Figure 10.



MCF-7 MTS at Day 10 (Pre-Dissociation)

Figure 10. MCF-7 spheroids pre-dissociation. MCF-7 MTS at day 10 of growth (7 days in 96-well culture plates and an additional 3 days in culture in spinner flasks). These spheroids are then used in the dissociation assay.

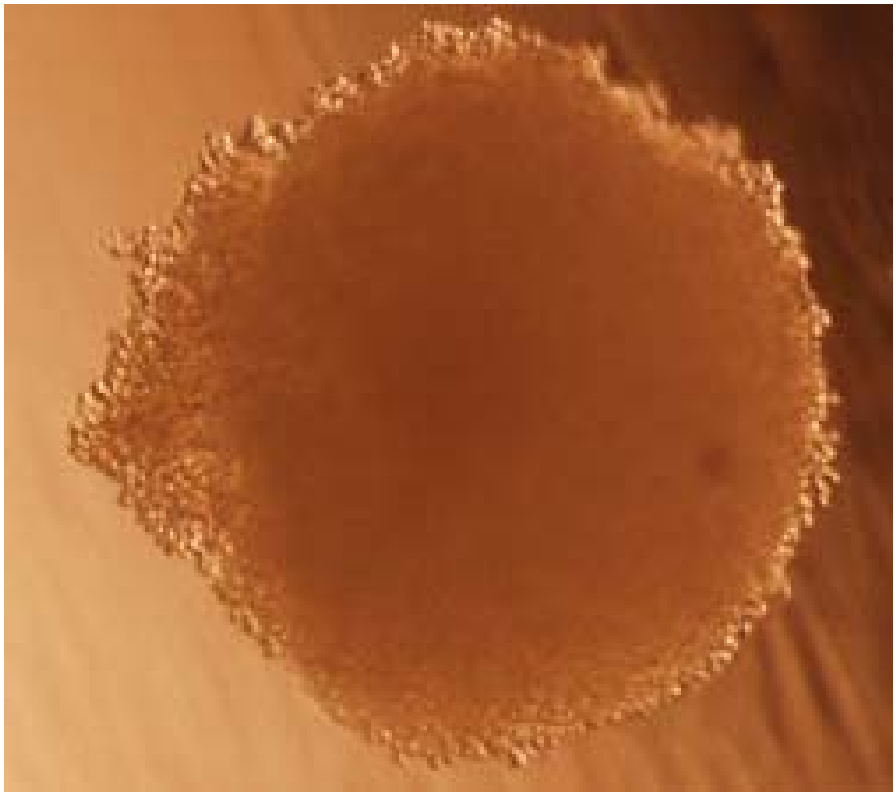
Figure 11.



MCF-7 MTS Post 10 3-minute Trypsinizations 10 (4X mag.)

Figure 11. MCF-7 MTS post dissociation. At dissociation step number 10, a sample of the spheroids was removed from the dissociation chamber and photomicrographs were taken.

Figure 12.



MCF-7 MTS Necrotic Core

Figure 12. MCF-7 spheroid necrotic core. MCF-7 MTS were subjected to 14 3-minute trypsinizations (continuous trypsin flow), leaving behind the central necrotic core.

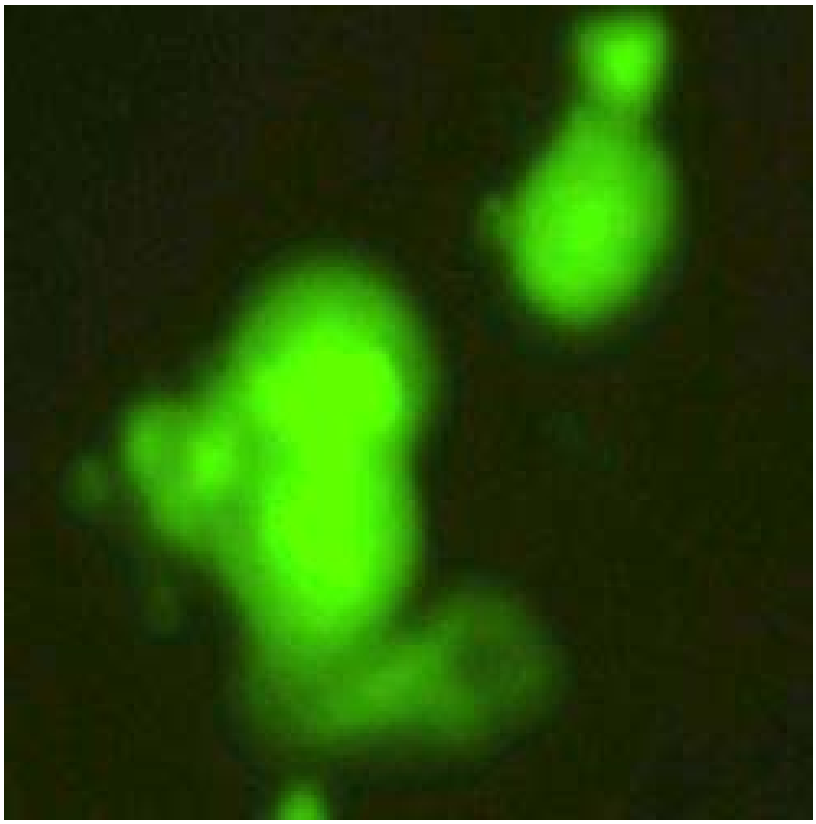
Table 1.

Experiment	Zones	RNA (ng/ml)
1	1	4.13
	2	41.77
	3	19.76
	4	7.53
2	1	0.42
	2	2.06
	3	12.75
	4	368.56
3	1	124.54
	2	66.31
	3	1093.69
	4	296.97
4	1	37.28
	2	34.72
	3	14.93
	4	42.66

MCF-7-MTS RNA concentrations.

Table 1. RNA concentrations of MCF-7 MTS proliferative zones. After combining the 14 dissociated cell fractions into 4 zones of decreasing proliferation (zone one being the highly proliferative zone and zone 4 being the least proliferative zone), RNA was isolated and quantified for use in gene expression studies.

Figure 13.



Fraction 6 of MCF-7 Dissociation

Figure 13. ***Ki-67 Expression in Fraction 6 of MCF-7 MTS.*** Ki-67 Expression in Fraction 6 of MCF-7 MTS. Cytospin slides were prepared from dissociated fraction 6. The slides were stained for Ki-67 antigen.

Figure 14.

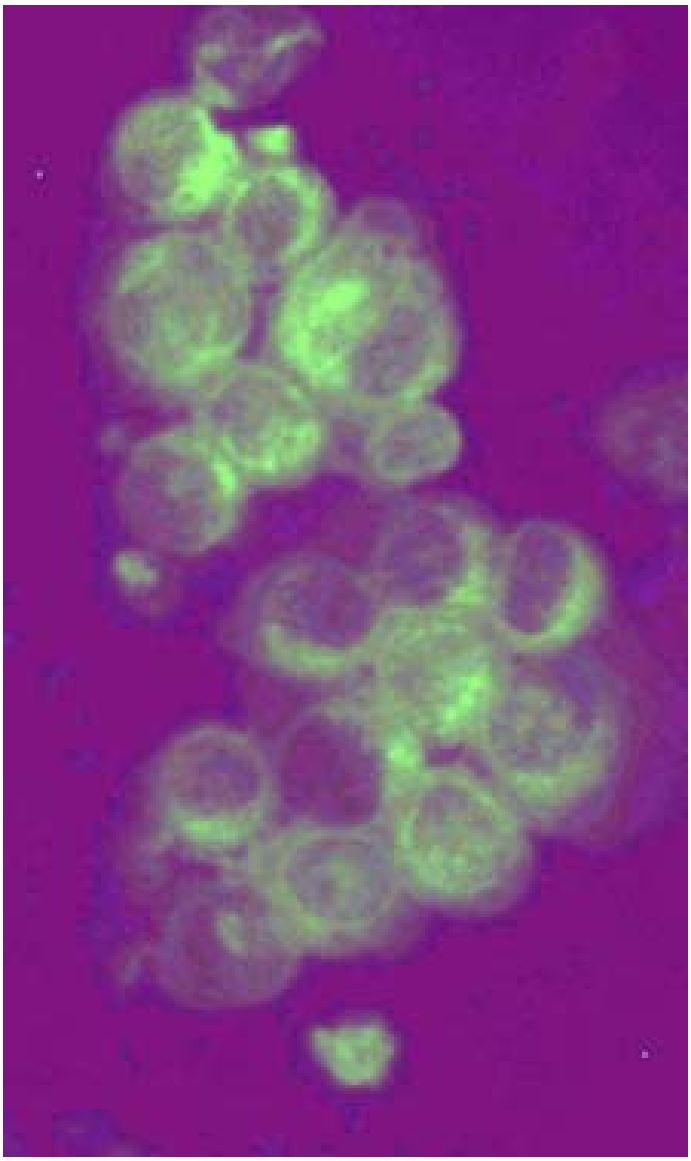


Figure 14. ***Ki-67 Expression in Fraction 11 of MCF-7 MTS*****.** Ki-67 Expression in Fraction 11 of MCF-7 MTS. Cytospin slides were prepared from dissociated fraction 11. The slides were stained for Ki-67 antigen.

Figure 15.

Figure 15. ***Ki-67 Expression in Fraction 15 of MCF-7 MTS*.** Ki-67 Expression in Fraction 15 of MCF-7 MTS. Cytospin slides were prepared from dissociated fraction 15. The slides were stained for Ki-67 antigen.